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NF-κB Binds P-TEFb to Stimulate Transcriptional Elongation by RNA Polymerase II

Matjaz Barboric,[1,3] Robert M. Nissen,[2] Satoshi Kanazawa,[1] Nabila Jabrane-Ferrat,[1] and B. Matija Peterlin[1,4]

[1] Howard Hughes Medical Institute
Departments of Medicine,
Microbiology, and Immunology
University of California at San Francisco
San Francisco, California 94143

[2] Center for Cancer Research
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

[3] Institute of Biochemistry
Medical Faculty of the University of Ljubljana
Vrazov trg 2
1000 Ljubljana
Republic of Slovenia

Summary

To stimulate transcriptional elongation of HIV-1 genes, the transactivator Tat recruits the positive transcription elongation factor b (P-TEFb) to the initiating RNA polymerase II (RNAPII). We found that the activation of transcription by RelA also depends on P-TEFb. Similar to Tat, RelA activated transcription when tethered to RNA. Moreover, TNF-α triggered the recruitment of P-TEFb to the NF-κB-regulated IL-8 gene. While the formation of the transcription preinitiation complex (PIC) remained unaffected, DRB, an inhibitor of P-TEFb, prevented RNAPII from elongating on the IL-8 gene. Remarkably, DRB inhibition sensitized cells to TNF-α-induced apoptosis. Thus, NF-κB requires P-TEFb to stimulate the elongation of transcription and P-TEFb plays an unexpected role in regulating apoptosis.

Introduction

The transcription of eukaryotic genes by RNAPII is a sophisticated biochemical process that is regulated at many levels. Human immunodeficiency virus 1 (HIV-1) is the paradigm for the regulation of transcriptional elongation (Taube et al., 1999). RNAPII initiates transcription in the HIV-1 long terminal repeat (HIV-1 LTR) but it elongates inefficiently, yielding predominantly short transcripts. Notably, the C-terminal domain (CTD) of the RPB1 subunit of RNAPII, which consists of 52 tandem repeats of the heptapeptide YSPTSPS, is hypophosphorylated. As a consequence, the paused RNAPII complex is associated with negative elongation factors. To overcome this inhibition, HIV-1 encodes the transactivator Tat, which is essential for optimal viral replication. Together with P-TEFb, which is composed of Cyclin T1 and Cdk9 (Price, 2000), Tat binds to the transactivation response element (TAR) RNA, which forms a stable structure at the start of all viral transcripts (Garber et al., 2000). In this way, Tat positions Cdk9 close to CTD, which becomes hyperphosphorylated (Zhou et al., 2000). The phosphorylation of CTD marks the critical step in the transition from initiation to elongation of transcription, thus allowing for productive viral replication. Indeed, potent inhibitors of P-TEFb, such as 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and flavopiridol, block the elongation of transcription as well as the production of viral particles in infected cells (Price, 2000; Chao et al., 2000).

The central member of the mammalian Rel/NF-κB family is NF-κB (Barkett and Gilmore, 1999). The major form of NF-κB exists as a heterodimer between RelA and p50. In the inactive cytoplasmic form, the RelA/p50 heterodimer binds to the inhibitor IκB protein. In response to a wide range of stimuli, the two conserved serine residues in the N-terminal domain of IκB become phosphorylated, resulting in its polyubiquitination and subsequent degradation (Karin and Ben-Neriah, 2000). Consequently, NF-κB translocates to the nucleus and stimulates the expression of its target genes.

Although P-TEFb is essential for the regulation of genes involved in the heat shock response (Lis et al., 2000) and in antigen processing and presentation (Kanazawa et al., 2000), our knowledge about the roles that P-TEFb plays in other cellular processes remains obscure. Since members of the Rel/NF-κB family regulate growth, transformation, and apoptosis of cells (Barkett and Gilmore, 1999), we were intrigued by the ability of flavopiridol to counteract proliferation and induce cell death (Chao et al., 2000). Further indications that NF-κB can regulate the elongation of transcription via P-TEFb come from studies where HIV-1 without a functional TAR can replicate in TNF-α-stimulated infected cells (Duh et al., 1989; Harrich et al., 1990). This cytokine causes the translocation of NF-κB to the nucleus and activation of its target genes. Indeed, effects of TNF-α on viral replication depend on the integrity of the two NF-κB binding sites within HIV-1 LTR. We therefore hypothesized that NF-κB uses the same machinery that has been hijacked by Tat to stimulate the transcriptional elongation of viral and cellular genes.

Results

NF-κB Requires P-TEFb to Activate Transcription

The promoter-proximal enhancer element within HIV-1 LTR contains two NF-κB binding sites that are recognized by RelA/p50 heterodimers and RelA homodimers (Jones and Peterlin, 1994). To determine if NF-κB regulates gene expression via P-TEFb, we used a plasmid reporter with four functional or nonfunctional NF-κB binding sites (pHIV-1-$(\kappa B)_4$-CAT or pHIV-1-$(m\kappa B)_4$-CAT, respectively) and examined the role of RelA and P-TEFb in this system (Figure 1A). Both plasmid targets also contained a TATA box and TAR from the HIV-1 LTR. The activation of pHIV-1-$(\kappa B)_4$-CAT by RelA would indicate that it can stimulate the release of paused RNAPII, which occurs at position +62 (Palangat et al., 1998). Moreover, its inhibition by a kinase-deficient Cdk9 protein (DNCdk9)

[4] Correspondence: matija@itsa.ucsf.edu

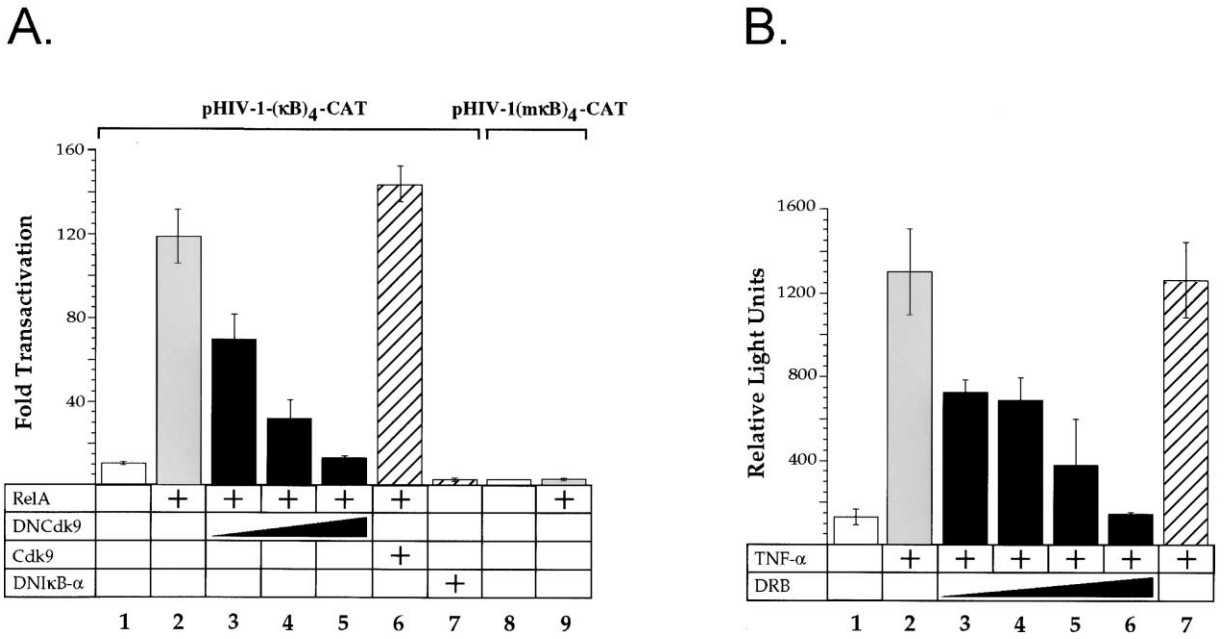

Figure 1. Obligatory Role for P-TEFb in NF-κB Transcription

(A) RelA activation of NF-κB responsive reporter gene is inhibited by DNCdk9. CV1 cells were transfected with pHIV-1-$(\kappa B)_4$-CAT (0.1 μg; bars 1–7) or pHIV-1-$(m\kappa B)_4$-CAT (0.1 μg; bars 8–9) as indicated on the top of the panel. The bottom part of the panel indicates proteins that were coexpressed with the plasmid reporters. In bars 2 to 6 and 9, cells were cotransfected with pCMVRelA alone (0.1 μg; gray bars 2 and 9) or together with increasing amounts of pCMVCdk9D167N, depicted as a black triangle (0.2, 0.4, 0.6 μg; black bars 3–5, respectively), or pCMVCdk9 (0.6 μg; hatched bar 6) in the presence of the indicated plasmid reporter. In bar 7, pCMVIκB-α (0.5 μg; hatched bar 7) was cotransfected together with the indicated reporter.

(B) The stable NF-κB-luciferase reporter gene is activated by TNF-α and inhibited by DRB. A549-k9 cells were treated with either TNF-α alone (5 ng/ml; gray bar 2), TNF-α and increasing levels of DRB, depicted as a black triangle (1, 3, 10, 30 μM; black bars 3–6), or TNF-α and DMSO (hatched bar 7). White bar 1 represents the activity of untreated cells.

would suggest that RelA requires P-TEFb to activate transcriptional elongation.

First, we cotransfected CV-1 cells with pHIV-1-$(\kappa B)_4$-CAT or pHIV-1-$(m\kappa B)_4$-CAT with a plasmid effector for RelA (pCMVRelA). Whereas the wild-type plasmid reporter was activated 118-fold by RelA, RelA had no effect on the basal activity of the mutant plasmid target (Figure 1A, compare bars 2 and 9). Importantly, the coexpression of increasing amounts of the DNCdk9 protein resulted in a dose-dependent repression of this activity (Figure 1A, compare bar 2 with bars 3 to 5). In contrast, when we coexpressed Cdk9 instead of the DNCdk9 protein, the activity of pHIV-1-$(\kappa B)_4$-CAT was unaffected. (Figure 1A, compare bars 2 and 6). Also, the DNCdk9 protein inhibited the activation of transcription by RelA from a plasmid reporter without TAR, demonstrating that TAR was not necessary for this effect (data not shown). Since the mutant IκB-α protein (DNIκB-α), which prevents the nuclear translocation of NF-κB, inhibited this activity (Figure 1A, compare bars 1 and 7), higher levels of basal transcription from pHIV-1-$(\kappa B)_4$-CAT (Figure 1A, compare bars 1 and 8) were due to the endogenous NF-κB.

To determine if functional interactions occur between endogenous NF-κB and P-TEFb, we used the A549-k9 cell line, which responds to TNF-α. This human lung carcinoma cell line carries a stably integrated luciferase reporter gene driven by three NF-κB sites (Nissen and Yamamoto, 2000). The stimulation of A549-k9 cells with 5 ng/ml of TNF-α increased the expression of luciferase 9-fold over basal levels (Figure 1B, compare bars 1 and 2). Next, we treated these cells with TNF-α together with increasing amounts of DRB and found that effects of TNF-α were repressed in a dose-dependent manner (Figure 1B, compare bar 2 with bars 3 to 6). Importantly, the cotreatment of cells with TNF-α and DRB at a concentration of 30 μM did not affect levels of RelA in the nucleus (data not shown). Mock-treated cells did not show any inhibition of transcriptional activity (Figure 1B, compare bars 2 and 7). Also, amounts of TNF-α employed were sublethal, and cells remained viable as measured by time-lapse videomicroscopy (data not shown). Overall, these studies demonstrate that NF-κB requires P-TEFb for efficient stimulation of transcription.

The Transactivation Domain of RelA Stimulates Transcription via RNA

HIV-1 LTR is a well-studied example of a natural RNA-tethering system. By modifying this promoter, several heterologous RNA-tethering systems have been created. One of them is presented in Figure 2A. The system consists of a plasmid target, pRRESCAT, made by grafting the high-affinity binding site for the HIV-1-encoded regulator of expression of virion proteins Rev (stem loop IIB, SLIIB) onto the stem of TAR (Tiley et al., 1992), and a series of plasmid effectors designed by fusing various test fragments to the Rev cDNA. The resulting artificial RNA-tethering system resembles Tat transactivation. In this way, one can ask if Rev chimeras stimulate the elongation of pRRESCAT transcription.

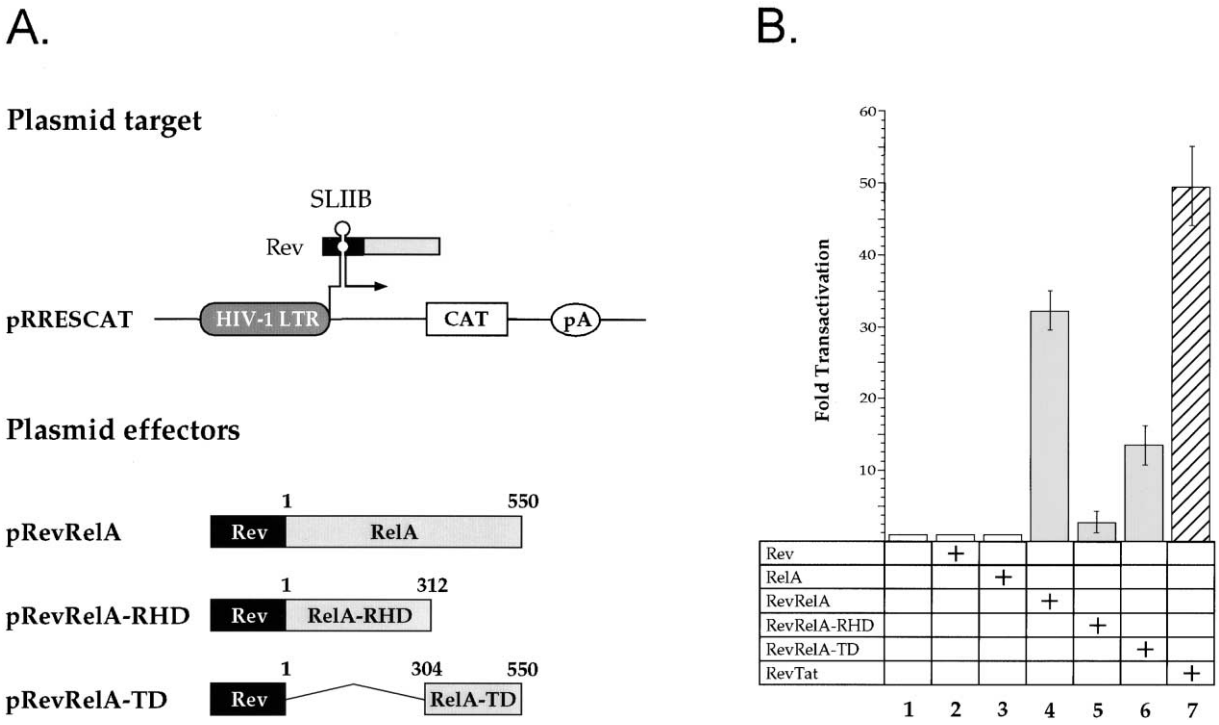

Figure 2. RelA Activates Transcription via RNA

(A) Schematic representation of plasmid target and effectors. pRRESCAT contains the high-affinity binding site for Rev (SLIIB). It directs the expression of the CAT reporter gene. The plasmid effectors pRevRelA, pRevRelA-RHD, and pRevRelA-TD consist of a cDNA coding for the Rev protein, presented in black, linked to a full-length RelA (amino acids 1 to 550), the NH_2-terminal Rel homology domain of RelA (amino acids 1 to 312), and the COOH-terminal RelA transactivation domain (amino acids 304 to 550), respectively.

(B) Transactivation domain of RelA is sufficient for the activation of pRRESCAT. CV-1 cells were transfected with pRRESCAT (0.1 μg; bars 1–7). The bottom part of the panel indicates the proteins that were coexpressed with the plasmid reporter from corresponding plasmid effectors as follows: pRev (0.5 μg; white bar 2), pCMVRelA (0.5 μg; white bar 3), the RevRelA chimeras (0.5 μg of each; gray bars 4 to 6), or pRevTat (0.5 μg; hatched bar 7).

To determine if NF-κB stimulates transcription via RNA, we examined the ability of the hybrid RevRelA, RevRelA-RHD, and RevRelA-TD proteins to activate pRRESCAT (Figure 2A). When we transfected CV-1 cells with pRRESCAT alone or together with plasmid effectors for Rev or RelA, we observed no activation of gene expression (Figure 2B, compare bars 1, 2, and 3). In contrast, RevRelA and RevRelA-TD chimeras activated pRRESCAT 32-fold and 14-fold over basal levels, respectively. Similarly, the hybrid RevTat protein, which served as the positive control, activated transcription 48-fold over the basal levels. (Figure 2B, compare bars 4 and 6 with bar 7). However, the RevRelA-RHD chimera was not active on pRRESCAT, suggesting that the transactivation domain of RelA is critical for its activity (Figure 2B, bar 5). Importantly, since the presence of increasing amounts of the DNCdk9 protein reduced this activity in a dose-dependent manner, effects of the hybrid RevRelA protein depended on P-TEFb (data not shown). We conclude that the transactivation domain of RelA is sufficient for activating transcription via RNA and that the P-TEFb kinase activity is critical for this effect.

RelA Associates with P-TEFb In Vivo and In Vitro

Results of the RNA-tethering experiment suggested that the transactivation domain of RelA recruits P-TEFb to the transcriptional machinery. Thus, one would expect that RelA interacts with P-TEFb. To examine this prediction, we coexpressed the HA-epitope-tagged Cdk9 protein (Cdk9-HA) with the pEFBOS empty vector (Figure 3A, lane 1) or with the HA-epitope-tagged RelA protein (RelA-HA) (Figure 3A, lanes 2 and 3) and incubated total cellular lysates with indicated antibodies. We detected the hybrid RelA-HA protein only in complexes immunoprecipitated with the antibody directed against Cdk9, whereas a nonspecific control antibody did not immunoprecipitate this chimera (Figure 3A, lanes 2 and 3). Protein levels of the hybrid Cdk9-HA protein were comparable in all experiments (Figure 3A, compare lanes 1, 2, and 3). We conclude that RelA interacts with P-TEFb in vivo.

CIITA, the master switch for transcription of MHC class II genes, binds to the cyclin box in Cyclin T1 (Kanazawa et al., 2000). To examine if RelA binds similarly to P-TEFb, we expressed deletion mutants of Cyclin T1 as GST-fusion proteins in *E. coli*. RelA was transcribed and translated in vitro using the rabbit reticulocyte lysate (Figure 3B). These proteins were then combined and the bound RelA was isolated using glutathione-Sepharose beads. As presented in the upper panel of Figure 3B, RelA was recovered with the hybrid GST-Cyclin T1 (1-551) as well as GST-Cyclin T1 (1-250) proteins (Figure 3B, lanes 2 to 3). In contrast, RelA did not bind to the GST-Cyclin T1 (233-551) fusion protein, which lacks the cyclin box (Figure 3B, compare lanes 1 with lanes 2 to

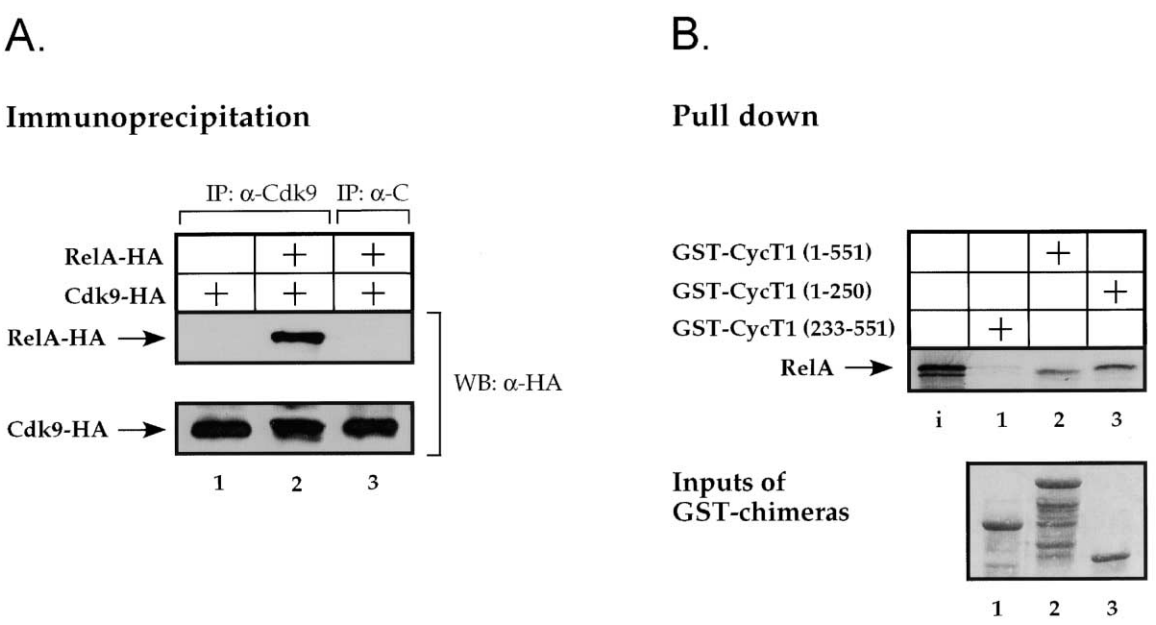

Figure 3. Association of Transcriptional Coactivator P-TEFb with RelA In Vivo and In Vitro

(A) RelA and P-TEFb associate in COS cells. HA-epitope-tagged Cdk9 was expressed alone (lane 1) or together with HA-epitope-tagged RelA (lanes 2 and 3), as indicated on the top of the panel. Total cell lysates were immunoprecipitated with the antibody directed against Cdk9 (α-Cdk9; lanes 1 and 2) or with a nonspecific control antibody (α-C; lane 3) and examined for the presence of RelA by Western blotting with the anti-HA antibody. The lower part of the panel shows the expression of Cdk9. Arrows to the left indicate the presence of HA-epitope-tagged proteins.

(B) RelA binds to the cyclin box of Cyclin T1. The upper part of the panel presents the pull downs. Binding reactions were performed as indicated above the autoradiograph. Lane i shows 10% of the input RelA, and lanes 1 to 3 represent pull downs. Arrow to the left points to the RelA band in the autoradiograph. The lower part of the panel shows the input of GST chimeras as visualized by Coomassie staining of the SDS-polyacrylamide gel.

3). Also, the input of GST-chimeras was comparable (Figure 3B, lower panel). Notably, when fused to the Gal4 DNA binding domain, Cyclin T1 derivatives, including the mutant Cyclin T1 (1-551) protein, activate transcription to similar levels when compared to the full-length Cyclin T1 protein (R. Taube et al., unpublished data). Thus, RelA binds the cyclin box of Cyclin T1.

TNF-α Triggers the Recruitment of P-TEFb to the NF-κB-Regulated IL-8 Promoter

To further analyze the association between P-TEFb and NF-κB in vivo, we examined whether individual components of both complexes were present together with DNA sequences of a well-described NF-κB-activated gene. For these experiments, we used A549-k9 cells to perform chromatin immunoprecipitation assays ([ChIP]; Orlando, 2000). We focused on the Interleukin-8 (IL-8) gene in A549-k9 cells because of its strict dependence on NF-κB and marked TNF-α induction.

First, we looked for the presence of RelA and components of P-TEFb at indicated chromatin regions of the IL-8 gene and compared it to the control U6 snRNA promoter (Figures 4A and 4B). In comparison to untreated cells, ChIP from TNF-α-treated cells with antibodies against RelA resulted in a 4-fold enrichment of sequences containing the IL-8 promoter. Cotreatment of A549 cells with TNF-α and DRB yielded the same 4-fold enrichment (Figure 4B, compare lane 4 with 5 and 6). In view of previous experiments where P-TEFb was required for effects of NF-κB, we envisioned that RelA and P-TEFb could occupy the chromatin region that includes NF-κB response elements. Indeed, Cdk9 and Cyclin T1 antibodies immunoprecipitated 3- and 7-fold more of the IL-8 promoter sequences, respectively, from TNF-α-treated cells when compared to untreated cells. Notably, this recruitment was unaffected by DRB (Figure 4B; compare lane 7 with lanes 8 and 9 and lane 10 with lanes 11 and 12). In control experiments, we did not observe any change in ChIP of the U6 snRNA promoter or a chromatin region 700 bp upstream of the IL-8 NF-κB response elements, indicating that TNF-α-dependent enrichment was sequence specific. Amounts of starting chromatin extracts were equal (Figure 4B, compare lanes 1 to 3). These results argue that subunits of NF-κB and P-TEFb are recruited specifically to the IL-8 promoter upon TNF-α treatment in vivo. That absolute values of Cdk9 and Cyclin T1 recruitment differed suggests that antibodies and corresponding epitopes perform with different efficiencies in the ChIP assay. Furthermore, DRB neither affected the signaling events triggered by TNF-α nor prevented the recruitment of either complex to the analyzed chromatin region. Most likely, DRB interfered with steps in transcription that follow the association of NF-κB and P-TEFb with the transcriptional machinery.

The Ability of NF-κB to Stimulate Elongation of Transcription Depends on P-TEFb

The hyperphosphorylated form of CTD is one hallmark of the elongating RNAPII (Price, 2000). Therefore, the

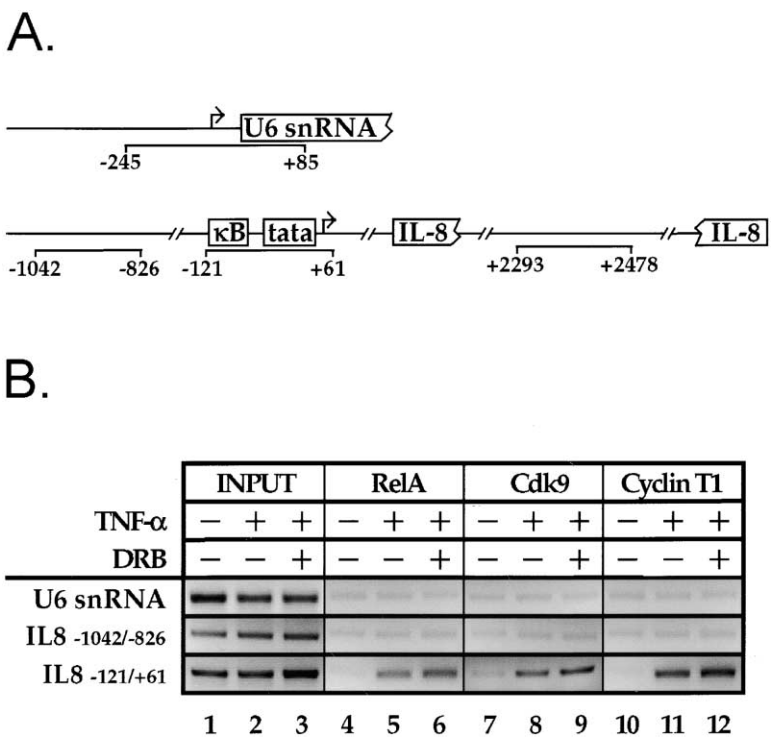

Figure 4. The Effect of TNF-α and DRB on Promoter Recruitment of P-TEFb and RelA

(A) Schematic representation of genomic regions that were analyzed by ChIP. IL-8 promoter region -121/+61, which contains functional NF-κB sites, and IL-8 coding sequence region +2293/+2478 were chosen as the probes. For controls, IL-8 promoter 5′-region -1042/-826 and the RNAPIII transcribed U6 snRNA region -245/+85 were chosen. For IL-8 region, NF-κB and TATA boxes are indicated. The arrows represent transcription starting points.

(B) The effect of TNF-α and DRB on Il-8 promoter occupancy by RelA and P-TEFb. A549-k9 cells were mock-treated or treated with 5 ng/ml of TNF-α alone or in the presence of 30 μM DRB as indicated. ChIP assays were performed with antibodies as indicated on the top of the panel. Chromatin immunoprecipitates were analyzed for the presence of U6 snRNA, IL-8 promoter 5′-region or IL-8 promoter region as indicated on the left. Left panel (INPUT, lanes 1 to 3) shows the starting A549-k9 chromatin extracts; right panels (RelA, lanes 4 to 6; Cdk9, lanes 7 to 9; Cyclin T1, lanes 10 to 12) show the enrichment of the IL-8 promoter region.

corecruitment of NF-κB and P-TEFb to the IL-8 promoter upon TNF-α treatment could result in the hyperphosphorylation of CTD. To address this hypothesis, we performed ChIP assays with monoclonal antibodies specific for the phosphoserine-2 (P-Ser2) and phosphoserine-5 (P-Ser5) heptapeptad repeats of CTD. We found that the treatment of A549-k9 cells with TNF-α stimulated P-Ser2 and P-Ser-5 occupancies of the IL-8 promoter 8-fold and 12-fold, respectively (Figure 5A, lanes 3 to 6). Control regions of the U6 snRNA gene were unaffected. Moreover, amounts of starting chromatin extracts were equal (Figure 5A, compare lanes 1 and 2). We conclude that the presence of P-TEFb on the IL-8 promoter correlates with the hyperphosphorylation of CTD of RNAPII.

Since NF-κB requires P-TEFb, inhibiting its kinase activity should impair the elongation of transcription by this activator. We therefore treated A549-k9 cells with TNF-α alone or in the presence of DRB and analyzed the chromatin regions depicted in Figure 4A for occupancy by RNAPII (Figure 5B). For these experiments, we used an antibody against the NH_2 terminus of the RNAPII RPB1 subunit. Since the antibody recognizes both the unphosphorylated and the phosphorylated forms of RNAPII, we could determine the recruitment of total RNAPII. Treatment of cells with TNF-α stimulated the RNAPII occupancy of the IL-8 promoter 7-fold and of the IL-8 gene coding sequence 12-fold (Figure 5B, compare lanes 4 and 5). Importantly, when we cotreated cells with TNF-α and DRB, the RNAPII occupancy of the IL-8 promoter did not change significantly. It was stimulated 10-fold. In contrast, these repressing conditions reduced the RNAPII occupancy of the IL-8 gene coding sequence region by nearly 75% to 3-fold (Figure 5B, compare lanes 5 and 6). As expected, the recruitment of RNAPII was promoter- and gene region-specific since control sequences 700 bp upstream of the NF-κB response elements were not enriched. Moreover, stimulating and repressing signals did not affect the ChIP of the control U6 snRNA promoter. Amounts of starting chromatin extracts were equal (Figure 5B, lanes 1 to 3). These results indicate that DRB inhibits NF-κB-activated transcription by interfering with a step that follows PIC assembly. Since P-TEFb acts on RNAPII in early elongation complexes, thus allowing its transition into an elongation-competent state, we conclude that DRB impairs the ability of NF-κB to stimulate the elongation of transcription.

Finally, we performed ribonuclease protection assays to demonstrate directly that RelA stimulates transcriptional elongation. For these experiments, we used plasmid effectors coding for Gal4-RelA and Gal4-Sp1 chimeras and examined their effects on pG6TAR. As presented in Figure 5C, pG6TAR contains six Gal4 DNA binding sites (UAS) upstream of the minimal promoter, which consists of three SP-1 binding sites, a TATA box, an initiator element, TAR, and the downstream CAT gene. We used two different RNA probes to independently evaluate levels of initiated and elongated transcripts. Whereas the promoter-proximal probe measured total levels of transcription, the distal probe measured only levels of elongated transcripts. It hybridized to mRNA species that elongated greater than 482 nucleotides downstream from the transcriptional starting site. When we coexpressed the Gal4-RelA chimera with pG6TAR in COS cells, we detected robust signals with proximal and distal probes as compared to the control Gal4-empty plasmid effector (Figure 5C, compare lanes 1 and 2). Importantly, levels of distal transcripts reached 52% of total levels of transcription. In sharp contrast, when we coexpressed the Gal4-Sp1 instead of the Gal4-RelA chimera, levels of distal transcripts reached only 5% of total transcription, which was comparable to basal lev-

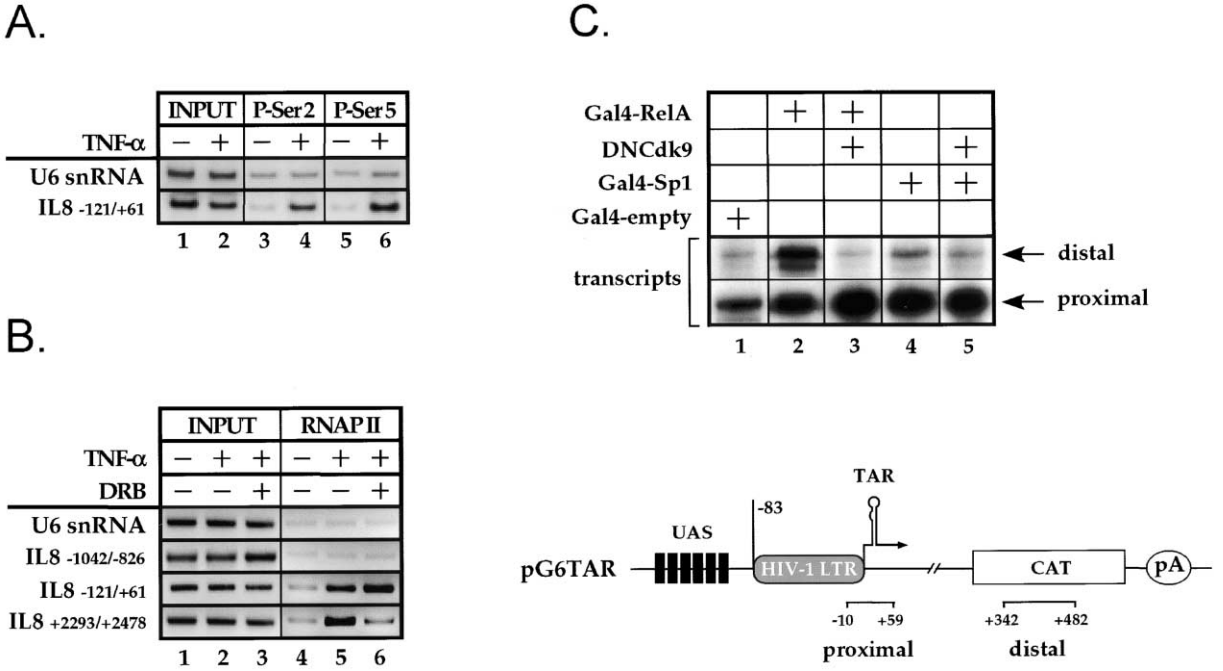

Figure 5. P-TEFb Is Critical for NF-κB to Activate Elongation of Transcription

(A) The effect of TNF-α on the phosphorylation of CTD of RNAPII at the IL-8 promoter. A549-k9 cells were mock-treated or treated with 5 ng/ml of TNF-α as indicated. ChIP assays were performed by using a P-Ser2 or a P-Ser5 antibody. Chromatin immunoprecipitates were analyzed for the presence of U6 snRNA or IL-8 promoter region as indicated on the left. Left panel (INPUT, lanes 1 and 2) shows the starting A549-k9 chromatin extracts; right panels (P-Ser2, lanes 3 and 4; P-Ser5, lanes 5 and 6) show the enrichment of the IL-8 promoter region.

(B) Elongation of IL-8 gene transcription is activated by TNF-α and inhibited by DRB. Chromatin immunoprecipitates were analyzed for the presence of U6 snRNA, IL-8 promoter 5′-region, IL-8 promoter region, or IL-8 coding sequence region as indicated on the left. Left panel (INPUT, lanes 1 to 3) shows the starting chromatin extracts; right panel (RNAPII, lanes 4 to 6) shows the enrichment of the IL-8 promoter or coding sequence regions.

(C) RelA stimulates transcriptional elongation. The upper part of the panel shows ribonuclease protection assays. The part of the panel above the autoradiograph indicates the proteins that were coexpressed together with pG6TAR. Arrows to the right of the autoradiograph point to protected proximal and distal transcripts. The lower part of the panel shows the schematic drawing of pG6TAR. Black rectangles (UAS) represent six Gal4 DNA binding sites. HIV-1 LTR (starting at the nucleotide -83) controls the expression of the CAT reporter gene. The positions of proximal and distal antisense RNA probes are presented.

els (Figure 5C, lane 4). Critically, the coexpression of the DNCdk9 protein and Gal4-RelA or Gal4-Sp1 chimeras reduced levels of distal transcripts to basal levels but did not affect the total levels of transcription (Figure 5C, lanes 3 and 5). We conclude that RelA stimulates transcriptional elongation and that P-TEFb is required for this effect.

Inhibition of P-TEFb Sensitizes Cells to TNF-α-Induced Apoptosis

The cytokine TNF-α is a well-characterized physiological inducer of NF-κB activity. Upon binding to its receptors (TNFR) on the cell surface, TNF-α triggers receptor multimerization and subsequent recruitment of adaptor and effector molecules to the initiating signaling complex inside the cell. On one hand, the recruitment of caspase-8 initiates the cellular apoptotic program. On the other, NF-κB becomes activated. Consequently, the transcription of antiapoptotic genes by NF-κB protects these cells from undergoing apoptosis (Barkett and Gilmore, 1999).

To elucidate the significance of P-TEFb in these cellular processes, we explored its involvement in the apoptotic process regulated by NF-κB. For these experiments, we treated A549 parental cells with various combinations of TNF-α and DRB and followed the fates of cells using time-lapse phase videomicroscopy (see supplemental movies at http://www.molecule.com/cgi/content/full/8/2/327/DC1). The treatment with TNF-α alone was not toxic to these cells (Figure 6A, open circles; Figure 6B, pictures 1 to 3; see Supplemental Movie 1 at http://www.molecule.com/cgi/content/full/8/2/327/DC1). Predictably, they behaved the same as mock-treated cells (data not shown). Next, we treated cells with DRB. We detected a limited number of apoptotic events, which started at 6 to 8 hr of treatment (Figure 6A, black rectangles; Figure 6B, pictures 4 to 6; see Supplemental Movie 2 at http://www.molecule.com/cgi/content/full/8/2/327/DC1). In sharp contrast, this interval and the number of detected cell death events was changed greatly when cells were cotreated with TNF-α and DRB. This cotreatment resulted in massive and rapid apoptosis, starting at 2 hr after the addition of both agents (Figure 6A, black circles; Figure 6B, pictures 7 to 9; see Supplemental Movie 3 at http://www.molecule.com/cgi/content/full/8/2/327/DC1). Morphological changes of dying cells resembled those characteristic of fibroblast apoptosis. A sudden onset of membrane

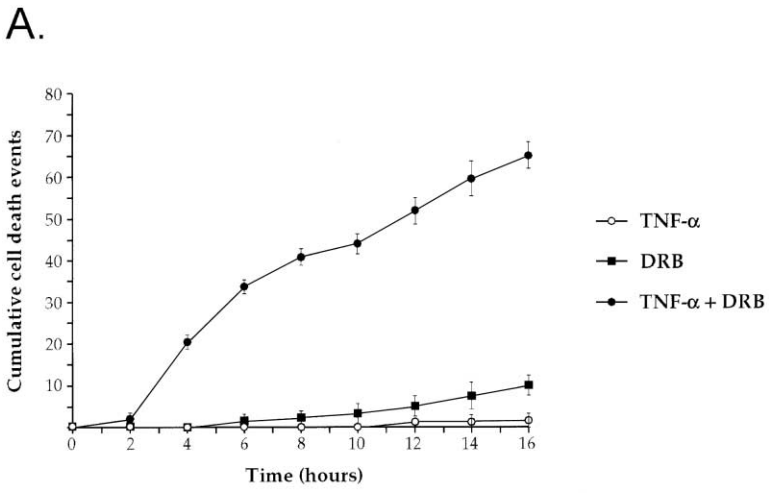

Figure 6. Survival of TNF-α-Treated A549 Cells Depends on P-TEFb

(A) The rate of apoptotic events after treatment with different combinations of drugs is shown. A549 cells were treated with 30 ng/ml TNF-α (open circles), 20 μM DRB (black rectangles), or 30 ng/ml TNF-α together with 20 μM DRB (black circles). The number of cell death events is expressed as a percentage of the total number of viable cells present in the entire frame at the beginning of the time-lapse experiment (~200 cells) and plotted against time. Data are representative of three independent experiments.

(B) Phase microscopy pictures of A549 cells taken at three different time points during the experiment. The left part of the panel presents combinations of agents used in each experiment. At time points indicated on the top part of the panel, the pictures of cells were taken by the digital camera. Data presented are representative of a typical experiment.

blebbing was followed by cell shrinkage and rounding of the cell body, nuclear pyknosis, and chromatin condensation (see supplemental data at http://www.molecule.com/cgi/content/full/8/2/327/DC1). Indeed, the co-treatment of cells with TNF-α and DRB in the presence of the permeable general caspase inhibitor benzyl-oxy carbonyl-Val-Ala-Asp (O-methyl) fluoromethylketone (zVAD.fmk) at a concentration of 25 μM effectively inhibited cell death (data not shown). Thus, upon activation of cells by TNF-α, NF-κB stimulates transcriptional elongation of antiapoptotic genes and the P-TEFb kinase activity is required for protecting cells from undergoing apoptosis.

Discussion

In this study, we provide evidence that a cellular transcription factor, NF-κB, uses P-TEFb to stimulate transcriptional elongation by RNAPII. First, we found that the kinase activity of P-TEFb was critical for NF-κB to activate transcription. Second, the transactivation domain of RelA was sufficient to activate transcription via RNA. Third, P-TEFb associated with RelA in cells and Cyclin T1 bound this subunit of NF-κB in vitro. Additionally, both complexes occupied the IL-8 promoter in TNF-α-treated cells, which resulted in the phosphorylation at serine-2 and serine-5 of CTD and subsequent elongation of transcription by RNAPII. Finally, the inhibition of P-TEFb by DRB sensitized A549 cells to TNF-α-induced apoptosis. We conclude that P-TEFb is an essential coactivator of NF-κB.

Findings of the present study add a new dimension to how RelA finalizes its transcriptional tasks. Thus, RelA not only promotes the initiation of transcription, but, through its association with P-TEFb, it also plays an essential role in the elongation of transcription. Impor-

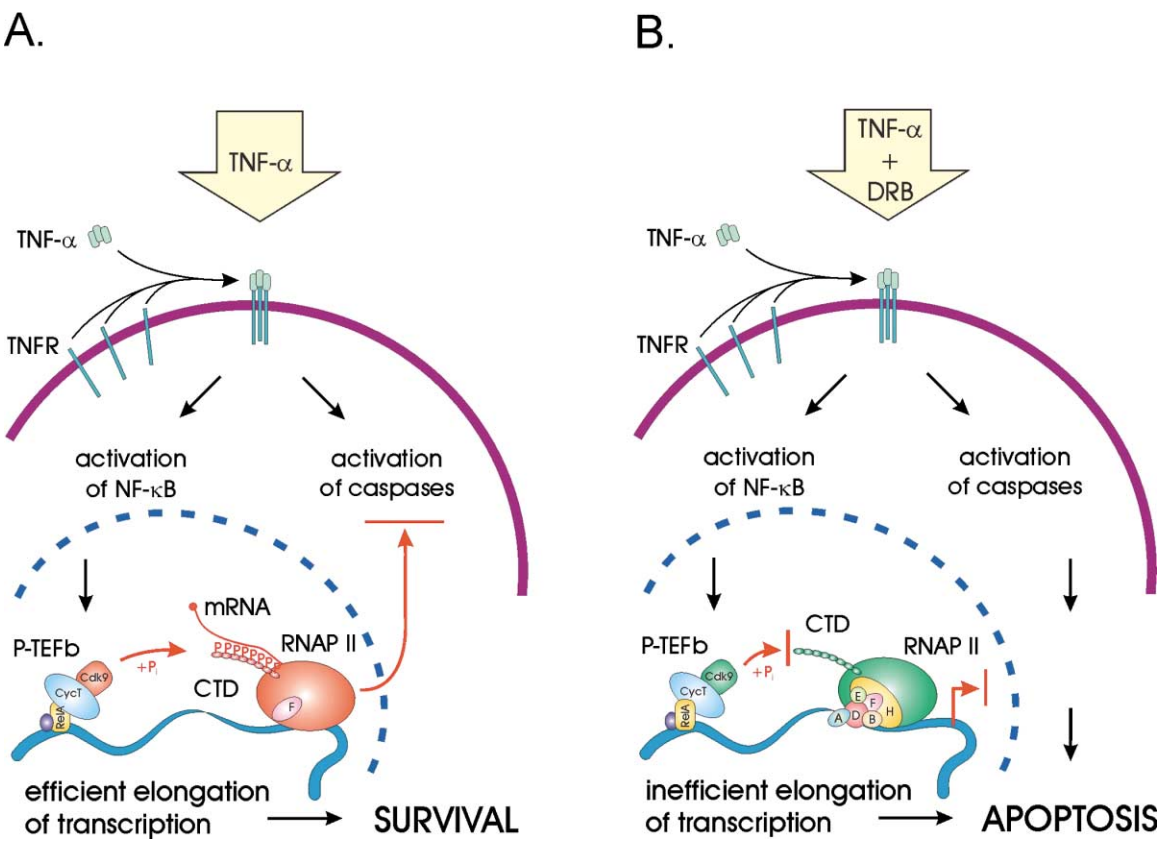

Figure 7. A Model for the Role of P-TEFb in NF-κB-Regulated Transcription and Apoptosis

Upon stimulating cells with TNF-α, NF-κB translocates from the cytoplasm to the nucleus. There, it binds to its response elements, stimulates PIC assembly and recruits P-TEFb, which then phosphorylates CTD of RNAPII. Efficient elongation of transcription of antiapoptotic genes interferes with the activated proapoptotic pathways and cells survive.

(B) DRB blocks the kinase activity of P-TEFb. As a consequence, the nuclear NF-κB protein does not activate the transcription of antiapoptotic genes. The TNF-α-induced death pathways are not counteracted by de novo synthesis of antiapoptotic proteins and cells undergo apoptosis. Black arrows represent pathways leading to the TNF-α-triggered TNFR multimerization and subsequent activation of NF-κB and caspases. Red arrows mark the ability of P-TEFb to phosphorylate CTD of RNAPII, stimulate the elongation of transcription, and block apoptosis. Blue curve represents DNA. The kinase-active Cdk9 is red, whereas the kinase-deficient Cdk9 is green. Likewise, the elongation-competent RNAPII is red, whereas the elongation-incompetent RNAPII is green. The general transcription factors $TF_{II}A$, B, D, E, F, and H are depicted in PIC.

tantly, the transactivation domain of RelA activated transcription when tethered artificially to the RNA element in a P-TEFb-dependent manner. Thus, RelA joins the small group of activators that stimulate the elongation of pRRESCAT transcription. They include Tat, VP16 (Tiley et al., 1992), Cyclin T1 (Bieniasz et al., 1999), Cdk9 (Fujinaga et al., 1998), and CIITA (Kanazawa et al., 2000). Although not determined yet for VP16, all of these proteins associate with or are part of P-TEFb. In contrast, transcriptional activators such as Sp-1 that stimulate only the initiation of transcription are inactive in this assay (Madore and Cullen, 1995). This latter group interacts with several factors that are required for PIC assembly as well as with the basal transcription factor TFIIH, whose CTD kinase activity is required for promoter clearance (Drapkin et al., 1994; Guzman and Lis, 1999; Maxon et al., 1994). Notably, we found that chimeric proteins between Rev and the three subunits of TFIIH, Cdk7, Cyclin H, and Mat1, do not activate pRRESCAT, consistent with their having roles in promoter clearance and not elongation of transcription (data not shown). Additionally, of the two kinases, only P-TEFb has the ability to confer elongating properties on early transcriptional complexes (Marshall et al., 1996). Thus, the RNA-tethering system is useful for assessing the ability of transcription factors to stimulate P-TEFb-dependent transcriptional elongation.

Chromatin immunoprecipitation and ribonuclease protection assays allowed us to evaluate directly the ability of RelA to affect the elongation of transcription. Consistent with results obtained with the RNA-tethering assay, only RelA but not Sp1 stimulated transcriptional elongation, which depended on P-TEFb. Importantly, we found that DRB, an inhibitor of P-TEFb and transcriptional activation by RelA, interfered with events that follow PIC assembly. Whereas DRB increased modestly the accumulation of promoter-bound RNAPII, it decreased drastically amounts of RNAPII downstream in the IL-8 gene, which is consistent with the formation and accumulation of promoter-paused transcriptional complexes. Thus, by inhibiting P-TEFb at the IL-8 gene, we were able to recapitulate the situation at the HIV-1 promoter (Taube et al., 1999) and at several mammalian genes, such as Ig-κ (Raschke et al., 1999) and c-*myc*

(Bentley and Groudine, 1986), where a strong transcriptional pause site delays the progression of RNAPII. In all these cases, the rates of initiation exceed the rates of elongation of transcription, thus accumulating stalled RNAPII. Interestingly, promoters of all these genes contain NF-κB binding sites. Notably, LPS, an inducer of NF-κB, activates Ig-κ gene transcription by improving the elongation properties of RNAPII (Raschke et al., 1999). Finally, we demonstrated that NF-κB and P-TEFb occupied the IL-8 promoter in TNF-α-treated cells, which was correlated with the hyperphosphorylated CTD of RNAPII. Importantly, P-TEFb phosphorylates serine-2 and serine-5 within the CTD heptapeptide repeats (Zhou et al., 2000). Interestingly, the glucocorticoid receptor specifically represses NF-κB activity by preventing the phosphorylation at serine-2 (Nissen and Yamamoto, 2000) which is critical for transcriptional elongation (Komarnitsky et al., 2000). Collectively, these observations support the notion that NF-κB stimulates transcriptional elongation via the phosphorylation of CTD mediated by P-TEFb.

Previous work demonstrated the pivotal role for RelA in stimulating PIC assembly by several different mechanisms (Nissen and Yamamoto, 2000, and references therein). Consistent with these reports, our ChIP experiments also demonstrated that RelA stimulates PIC assembly in TNF-α-treated cells. Remarkably, CIITA has acquired transcriptional activities that are very reminiscent of RelA (Harton and Ting, 2000). Similar to RelA, CIITA attracts the core machinery via its binding to general transcription factors, it associates with CBP/p300 to increase accessibility of chromatin-condensed templates, and it recruits P-TEFb to stimulate transcriptional elongation. It is of note that both RelA and CIITA possess acidic transcriptional activation domains. Thus, the growing evidence suggests that an additional functional interaction exists for this class of eukaryotic transcription factors: besides recruiting and stabilizing various components of PIC to initiate transcription, their interaction with P-TEFb promotes the elongation of transcription.

The demonstration that NF-κB interacts with P-TEFb resolves an enigma concerning how the very first rounds of HIV-1 transcription occur prior to the synthesis of Tat. We suggest a scenario where NF-κB binds to HIV-1 LTR, recruits P-TEFb, and phosphorylates CTD. Thus, NF-κB substitutes for the lack of transcriptional elongation effects in the absence of Tat. In favor of this notion, cells stimulated by TNF-α support the replication of HIV-1 in a TAR-independent and NF-κB-dependent manner (Duh et al., 1989; Harrich et al., 1990).

An increased understanding of the detailed molecular events involved in proviral activation could provide further insights into the generation and perhaps the eradication of latent reservoirs of HIV-1. Despite many successes of the highly active antiretroviral therapy (HAART), the eradication of HIV is still beyond our reach due to the persistence of latently infected cells. They can reestablish the production of HIV-1 upon cellular activation. Notably, the activation of latently infected peripheral blood mononuclear cells from an asymptomatic infected individual stimulates transcriptional elongation of HIV-1 (Adams et al., 1994). Thus, these observations reinforce the idea that the capacity of NF-κB to associate with P-TEFb and stimulate transcriptional elongation of latent proviruses performs a critical role in reinitiating viral replication.

Importantly, we found a previously unrecognized role for P-TEFb in determining cell fate. Our experiments demonstrate that the efficient elongation of transcription by NF-κB represents an obligatory element in the protection of cells from undergoing apoptosis. In fact, RelA induces many antiapoptotic genes (Barkett and Gilmore, 1999). These insights led us to propose the scenario outlined in Figure 7. Interestingly, in serum-deprived NIH 3T3 cells, the ectopic expression of the TIP30 CTD kinase increases the expression of a subset of proapoptotic genes in a kinase-dependent manner (Xiao et al., 2000). Therefore, it is tempting to speculate that the phosphorylation of CTD of RNAPII might represent one of many significant crossroads at which cell fates are defined.

In summary, this study demonstrates that NF-κB associates with P-TEFb to stimulate the elongation of transcription by RNAPII. Also, it provides an explanation for how HIV-1 subverts the transcriptional activities of eukaryotic cells during early events in its replicative cycle. Moreover, its critical role in protecting cells from apoptosis indicates that P-TEFb and transcriptional elongation play key roles in cellular proliferation and cancer. The importance of different P-TEFb complexes and how many different transcriptional units are regulated by P-TEFb remains an interesting enigma for the future. The observations that P-TEFb localizes to more than 200 distinct sites on *Drosophila* polytene chromosomes (Lis et al., 2000) and that Cyclin T1 can activate transcription via enhancer elements (R. Taube et al., unpublished data) suggest the global importance for P-TEFb in regulating transcriptional elongation in eukaryotic cells.

Experimental Procedures

Cell Culture and Cell Lines

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 mM L-glutamine, and 50 μg each of penicilin and streptomycin per ml. All cells were grown at 37°C with 5% CO_2. A549-k9 cells carrying stably integrated NF-κB-responsive luciferase reporter genes were described previously (Nissen and Yamamoto, 2000).

Plasmid DNAs

Plasmid reporters pHIV-1-$(\kappa B)_4$-CAT, pHIV-1-$(m\kappa B)_4$-CAT, pRRESCAT, and pG6TAR were described previously (Madore and Cullen, 1993; Ghosh et al., 1993). Plasmids coding for Cdk9 (pCMVCdk9), DNCDK9 (pCMVCdk9D167N), pRevTat, and GST-Cyclin T1 fusion proteins were described previously (Madore and Cullen, 1993; Fujinaga et al., 1999). The plasmids pCMVRelA, pCMVIκB-αS32/36A, pGal4-RelA, and pGAL4-Sp1 were gifts from Dr. Nissen. The plasmid pcDNA3.1-p65 was a gift from Dr. Okamoto. The plasmids pMTX-89 and pMTX-147 were a gift from Dr. Karn and were described previously (West and Karn, 1999). The *E. coli* expression vector pGEX-RelA for the GST-RelA fusion protein was described previously (Nissen and Yamamoto, 2000). To construct pEFNRev, a cDNA coding for Rev protein from HIV-1 was inserted into NcoI-EcoRI sites of the modified pEFBOS vector. Plasmid coding for RevRelA, RevRelA-RHD, and RevRelA-TD chimeras were made in the pEFNRev expression vector by cloning amplified PCR products into EcoRI sites. To construct HA epitope-tagged pEFRelA, RelA was excised from pRevRelA using EcoRI restriction enzyme and inserted into EcoRI site of the modified pEFBOS vector.

Chemicals

DRB was obtained from Sigma (St. Louis, MO). zVAD.fmk was obtained from Enzyme Systems Products (Dublin, CA). TNF-α was obtained from Roche Molecular Biochemicals (Indianapolis, IN).

Transient Transfection, CAT Assay, and Luciferase Reporter Gene Assay

CV-1 cells and COS cells were transiently transfected with Lipofectamine according to the manufacturer's instructions (Gibco-BRL, Rockville, MD). Chloramphenicol acetyltransferase (CAT) enzymatic assays were performed as described (Fujinaga et al., 1998). For luciferase reporter gene assays, A549-k9 cells were plated in 24-well plates (1-2 × 10^5 cells per well) approximately 12 hr prior to transfection. After treatment with various drug combinations for 8 hr, cells were harvested and assayed for luciferase activity using the Enhanced Luciferase Assay Kit (Pharmingen, San Diego, CA) as described (Nissen and Yamamoto, 2000). In all transfections, the amount of DNA was equilibrated with a corresponding empty vector. A cytomegalovirus-β-galactosidase plasmid reporter (Gibco-BRL) was used to monitor transfection efficiency. The activity of the reporter plasmid alone is given as one. Data are representative of three independent transfections, which were performed in duplicates. Error bars give standard errors of the mean.

Immunoprecipitation Assay and Western Blot Analysis

Immunoprecipitation assay was performed as described (Kanazawa et al., 2000). 3 μg of pEFRelA-HA, 0.5 μg of pCMVCDK9-HA, or the corresponding amount of empty plasmid vector was cotransfected into COS cells as above. 10 μl of anti-Cdk9 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or a nonspecific control rabbit serum was used. Western blotting was performed according to standard protocols.

Ribonuclease Protection Assays

COS cells were seeded into 150 mm in diameter petri dishes (2 × 10^6 cells per plate) approximately 12 hr prior to transfection. 3 μg of pG6TAR reporter plasmid, 3 μg of pSG424 (Gal4-empty), 3 μg of pGal4-RelA, 3 μg of pGal4-Sp1, 9 μg of pCMVCdk9D167N, or the corresponding amount of empty plasmid vector was transfected into the cells. Cells were harvested 24 hr posttransfection, and total RNA was extracted using TRIzol reagent (Gibco-BRL, Rockville, MD). Template DNAs for the preparation of antisense probes were generated as described (West and Karn, 1999). Antisense probes were prepared using MAXIscript T3 Kit (Ambion, Austin, TX) according to manufacturer's recommendations. The ribonuclease protection assays were performed on 20 μg total RNA and 20 000 c.p.m. of antisense probes using RPA III Ribonuclease Protection Assay Kit (Ambion, Austin, TX) according to manufacturer's recommendations.

In Vitro Binding Assays

GST-Cyclin T1 fusion proteins were expressed in *E. coli* BL21(DE3)-pLysS competent cells (Novagen, Madison, WI) and purified as described (Kanazawa et al., 2000). Twenty micrograms of each chimera was incubated with 15 μl of ^{35}S-labeled RelA, which was transcribed and translated in vitro using the TnT T7 coupled reticulocyte lysate system as instructed by the manufacturer (Promega, Madison, WI). Each binding reaction was perfomed in 100 μl of binding buffer (20 mM HEPES [pH 7.9], 1% Triton X-100, 20 mM DTT, 0.5% BSA, and 100 mM KCl) for 3 hr at 4°C. After the binding, glutathione Sepharose beads were added and washed with the same buffer without BSA three times. Bound proteins were eluted by boiling in the SDS sample buffer, resolved by SDS-PAGE on a 10% gel, and revealed by autoradiography.

Chromatin Immunoprecipitation Assays

The ChIP assay was performed essentially as described (Nissen and Yamamoto, 2000) with minor modifications. Approximately 5 × 10^8 adherent A549-k9 cells were treated for 5 hr with various drug combinations before crosslinking. To cleared chromatin extracts, either 2 μg of RelA (Santa Cruz Biotechnology, sc-109, Santa Cruz, CA), Cdk9 (Santa Cruz Biotechnology, sc-8338, Santa Cruz, CA), total RNAPII (Santa Cruz Biotechnology, sc-899, Santa Cruz, CA), or 20 μl of rabbit polyclonal Cyclin T1 antibody (gift from David H. Price) was added. For the CTD phosphoserine-2 and phosphoserine-5 ChIP assay, chromatin extracts were prepared with all buffers including the general phosphatase inhibitor Na-pyrophosphate (10 mM [pH 8]). To cleared chromatin extracts, either 5 μl of the CTD phosphoserine-2 specific monoclonal antibody H5 (BAbCO) or 10 μl of the CTD phosphoserine-5 specific monoclonal antibody H14 (BAbCO) was added. 50 μl PCR reactions were programed for 30 cycles with 1/8 of precipitated DNA. Titrations were performed to ensure a linear range of amplification. One-third of each PCR reaction was electrophoresed on a 2% 1× TAE agarose gel and quantitated using the Multi analyst program. The IL-8 gene promoter and coding sequence intensities were first normalized by dividing them by the internal control intensity of the U6 snRNA gene. Fold induction represents the ratio between the normalized intensities for the treated in comparison to the untreated experimental sample. PCR primer sets for the human IL-8 gene promoter region -121/+61, human IL-8 upstream region -1042/-826, and human U6 snRNA gene promoter region -245/+85 were the same as described (Nissen and Yamamoto, 2000). The human IL-8 gene coding sequence region +2293/+2478 was amplified with the PCR primer pairs 5′-GCCA TAAAGTCAAATTTAGCTGGAA-3′ and 5′-GTGCTTCCACATGTCCT CACA-3′.

Time-Lapse Videomicroscopy

Acquisition of time-lapse phase-contrast images has been described previously (Kauffmann-Zeh et al., 1997). The cells were plated onto six-well dishes at 50% confluency. Twelve hours later, the cells were treated with the drugs as indicated, transfered to a six-well environmental chamber, and observed with a Zeiss inverted microscope using 10× objective with LUDL-controlled *XYZ* movement operated by OpenLab software version 2.1.1. The cells were followed by time-lapse phase videomicroscopy at a rate of 12 frames per hour and scored for apoptosis. At the end of each 2 hr interval, the total number of apoptotic events thus far was summed and expressed as a percentage of the total number of viable cells present in the entire frame at the beginning of the experiment. Apoptotic cell death events were scored midway between the last appearance of normality and the point at which the cell became fully detached and rounded.

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